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(54) Titre: MUTANT ATTENUE DE LISTERIA MONOCYTOGENES; SOUCHE RECOMBINANTE DE LISTERIA MONOCYTOGENES, UTILISATION COMME VECTEURS HETEROLOGUES D'ANTIGENE VACCINAL ET UTILISATION COMME VACCIN OU COMPOSITION DIAGNOSTIQUE

(57) Abstract

The invention discloses an attenuated mutant of *Listeria monocytogenes* incorporating in the act A gene or in its promotor a mutation capable of blocking or modifying the expression of the protein coded by the act A gene. This mutant can be used as a livin g vector for the expression of an heterologous ADN, particularly a gene coding for a viral, bacterial or parasitic protector antigene which is the target of T cells of subclass CD8. The recombinant mutant strains thus obtained may be used as a vaccine or diagnostic composition for checking the protection state of a host.

(57) Abrégé

L'invention a pour objet un mutant atténué de *Listeria monocytogènes* comportant dans le gène act A ou dans le promoteur de celui-ci une mutation apte à bloquer ou modifier l'expression de la protéine codée par le gène act A. Ce mutant peut être utilisé en tant que vecteur vivant pour l'expression d'un ADN hétérologue, notamment d'un gène codant pour un antigène viral, bactérien ou parasitaire protecteur cible de lymphocytes T de la sous-classe CD8. Les souches mutantes recombinantes ainsi obtenues ont des applications en tant que vaccin ou composition de diagnostic pour le contrôle de l'état de protection d'un hôte.

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Attenuated mutant of Listeria monocytogenes; recombinant strain of Listeria monocytogenes, use as heterologous vectors of vaccinal antigen and use as vaccine or diagnostic composition.

This invention relates to an attenuated mutant strain of Listeria monocytogenes and its immunotherapeutic and diagnostic applications, especially for the production of a recombinant strain usable as a vaccine.

Listeria monocytogenes is a non-spore forming, gram-positive facultative aerobic bacillus, ubiquitous in the environment and responsible for human and animal listeriosis. The disease manifests itself in opportunistic infections, either as meningitis and/or encephalitis, septicemias, or abortions, with a high mortality rate in newborns and in adults whose defense mechanisms are weakened by pregnancy, immunosuppression caused by treatment, an underlying illness, or old age. Listeriosis may also affect apparently healthy subjects.

Listeria monocytogenes is capable of infecting, both in vivo and in vitro, a wide variety of cellular types, especially macrophages, fibroblasts, epithelial cells, and enterocytes.

After its penetration into the infected cell, the bacterium lyses the phagosome membrane using a hemolysin that it secretes. At the end of this stage, the bacterium is in the cytoplasm of the host cell.

In addition, Listeria monocytogenes is characterized by its ability to propagate in tissues by direct infection from cell to cell without leaving the cytoplasm (Racz et al., 1970 (9)).

A short time after it enters the host cell, the bacterium is surrounded by filamentous actin (F-actin), which is later rearranged in a "comet tail" behind the

bacterium in the opposite direction of the movement (Tilney et al., 1989, (13); Mounier et al., (1990) (1). The polymerized actin is made up of short microfilaments, randomly oriented, which differ from the long F-actin filaments normally observed in muscle cells.

The bacteria are motile and leave "comet tails" of F-actin that are several μm in length behind them. Some of them are incorporated into finger-shaped cytoplasmic protuberances that may be internalized by the neighboring cells. The two plasmic membranes surrounding the bacterium are then lysed. Once in the cytoplasm of the new host cell, the bacterium can reproduce and begin a new spreading cycle.

During this spreading process, the Listeria monocytogenes cells are protected from the immune system of the host and the cell-to-cell spread consequently represents a key virulence factor.

By isolating and analyzing a Tn 917-lac mutant incapable of spreading from cell to cell, the inventors were able to identify a Listeria monocytogenes protein involved in the accumulation of actin induced by the bacteria.

The encoding gene for this protein named act A, is part of an operon (Mengaud et al., 1991 (b) (8)), of which the complete nucleotide sequence has recently been described (Vazquez-Boland et al., 1992 (12)).

The object of this invention is thus a strain of Listeria monocytogenes of attenuated virulence, characterized by the fact that it comprises, in the act A gene or in the promoter of that gene, a mutation capable of blocking or substantially modifying the expression of the protein encoded by the act A gene.

The mutation may be carried out in accordance with known techniques, especially by inserting into the act A gene or its promoter a sequence of one or more bases, preferably a stable transposon, deletion of one or more bases, mutations such as mutations by directed mutagenesis, for example by PCR and especially missense mutation.

The mutation may especially be carried out through insertion of a transposon, such as the Tn 917-lac transposon, as described by Mengaud et al., 1991 (a) (7).

The mutation is preferably carried out in the encoding DNA fragment for the peptide sequence with repeated patterns included between amino acids 235 to 315, 350 to 360, 367 to 385, and 389 to 393 of peptide sequence SEQ ID No. 1.

Another advantageous mutation site, especially for insertion, is located downstream from adenosine in position 497 of nucleotide sequence of the act A gene.

That position corresponds to the position between amino acids 61 and 62 of peptide sequence SEQ ID No. 1.

An especially preferred strain as claimed in the invention is the Listeria monocytogenes strain LUT 12 deposited in the Collection Nationale de Cultures de Micro-organismes (CNCM) [National Collection of Micro-Organism Cultures] on January 30, 1992 under the number I-1167.

The mutants as claimed in the invention are capable of providing protection against a subsequent infection by a pathogenic strain of Listeria monocytogenes on hosts to which they have been administered.

Therefore, the object of the invention is also a human or veterinary vaccine comprising an attenuated strain of Listeria monocytogenes, such as defined previously, as an active ingredient.

This vaccine is capable of providing effective protection against listeriosis to humans or animals, especially bovines and ovines.

The immune response generated by the administration of an attenuated mutant as defined involves the proliferation essentially of T lymphocytes of the CD8 sub-class.

T lymphocytes of the CD8 sub-class are activated by peptides bound to MHC (major histocompatibility complex) class I antigens generated by the proteolysis of synthesized or released proteins in the cytoplasm of a cell that has the antigen.

Thus, the attenuated Listeria monocytogenes mutants as claimed in the invention are capable of stimulating the immune system by using MHC class I molecules.

Consequently, it is possible, by transforming Listeria monocytogenes by means of an appropriate plasmid, to introduce a heterologous gene from any organism and to use the recombinant strains obtained as a system of expressing heterologous DNA.

Thus, the object of the invention is also a recombinant mutant of Listeria monocytogenes, characterized by the fact that it comprises a heterologous DNA, either inserted into the genome of an attenuated mutant as defined previously, or carried by a plasmid that replicates in the attenuated mutant.

The heterologous DNA preferably consists of a heterologous encoding gene for a protective antigen that is the target of sub-class CD8 T lymphocytes.

This antigen may be of a bacterial (for example, of mycobacteria), parasitic (for example, of Leishmania, Trypanosoma, or Toxoplasma), or viral (influenza virus, lymphocytic choriomeningitis virus (LCMV) or AIDS virus (HIV)) origin.

An especially interesting recombinant mutant of Listeria monocytogenes comprises the encoding genes for the HIV gag antigen or nef antigen, or all or part of the HIV-1 coating gp 120 or HIV-2 coating gp 140 or by a peptide as defined in US-4 943 628.

The construction of the recombinant mutant may be carried out by transforming an attenuated mutant as defined above, in particular the mutant LUT 12 with an appropriate plasmid, and, for example, electroporation.

Advantageously, the cloning of the heterologous DNA shall be carried out in E. Coli and an E. Coli - Listeria monocytogenes shuttle plasmid will be used to carry out the transformation.

As plasmids, pMKA 4 (Sullivan et al., (14)) or PHT 320 (Leredus et al., (15)) can be cited.

To make possible the expression of the gene of interest (heterologous gene), it is advantageous to insert a strong Listeria promoter, such as the hly promoter, upstream of the gene of interest.

So that the translation product of the gene of interest may be secreted, it is preferable to fuse the gene of interest at the beginning of hly in order to use the signal sequence of the listeriolysin O to release the protein of interest encoded by the heterologous gene in the cytoplasm of a host cell.

The recombinant mutants of Listeria monocytogenes as defined above are advantageously suitable for the preparation of a recombinant human or veterinary vaccine against an infection caused by a micro-organism that produces an antigen corresponding to the protein encoded by the heterologous DNA inserted in the genome of recombinant Listeria monocytogenes.

The vaccines as claimed in the invention may be administered intravenously, subcutaneously, intramuscularly, or orally.

An appropriate dose ranges from 5.10^4 and 10^9 cells/kg of weight.

This dose varies as a function of the route of administration as well as the sensitivity of the host.

The administration is preferably repeated in order to provide the host with effective protection.

The recombinant mutants of Listeria monocytogenes defined above are also suitable for the preparation of a diagnostic composition intended to check the protection status of a human or animal host against an infection caused by a micro-organism that produces an antigen corresponding to the protein encoded by the heterologous DNA inserted in the genome of recombinant Listeria monocytogenes or expressed in that strain when carried by a plasmid.

It will be sufficient to locally inject the diagnostic composition as claimed in the invention, for example, subcutaneously, and to observe after a certain latency period

whether or not an inflammatory response takes place, in the manner of the tuberculin test used to check the protection status of a host against the tubercle bacillus.

Below we will describe the obtaining of the mutant strain LUT 12 of Listeria monocytogenes as well as its properties, referring to the attached figure that represents:

A: the lecithinase operon of Listeria monocytogenes (Vasquez - Boland et al., 1992 (12) with the position of the transposon in the mutant L 12.

B: the amino acid sequence of the protein encoded by the act A gene.

The heavy black lines represent genes of which the products were characterized (mpl: metalloprotease, Domann et al., 1991, (3) act A: (this invention), plc B: lecithinase) ORFX, ORFY, and ORFZ are open reading frames.

P: indicates the promoter; the dashed lines the transcription product and a potential transcription termination signal.

The potential signal sequence and the membrane-spanning segment are underlined. The area of repeated patterns is circled. The arrow corresponds to the insertion of Tn 917-lac in the act A gene of the mutant LUT 12.

The numbering begins at the NH_2 end of the mature protein. The residues determined by microsequencing the 90 kDa band are printed in boldface and marked with an asterisk.

I - General cloning and DNA analysis techniques

All the cloning and analysis techniques were carried out in accordance with the standard protocols (Sambrook et al. 1989 (10)) or according to the manufacturer's instructions.

The chromosomal DNA of Listeria monocytogenes was prepared as described by Mengaud et al., 1991 (b) (8). The probes for Southern blot were prepared by PCR, purified from agarose gels using the Geneclean kit (Bio 101, Inc., La Jolla, CA), and labeled using the Amersham Multiprime system.

The hybridizations according to Southern were carried out with the rapid hybridization system (Amersham) on Nylon N membranes (Amersham) in a Hybaid hybridization oven.

II - Isolation of the strain LUT 12 and determination of the insertion point of the transposon

A bank of mutants Tn 917-lac, produced from the wild-type strain LO28 and from plasmid p TV32 carrying the transposon Tn 917-Lac as described by Mengaud et al., 1991 a (7) was screened on egg yolk agar plates prepared from fresh egg yolk diluted 1:2 in a solution of NaCl 150 mM and the addition of 12.5 ml of this mixture to 250 ml of brain-heart infusion (BHI) agar at 56°C.

A lecithinase-negative mutant which does not produce opacification of the egg yolk even after prolonged incubation and which shows a wild-type phenotype for all the other traits examined was called LUT 12.

A - Biological characteristics of this mutant

This mutant had both a hemolytic activity and an in vitro growth rate identical to the wild type, but proved to be of very attenuated virulence in mice.

TOXICITY

The LD_{50} was higher by a factor of 4 log 10 than the LD_{50} of wild-type bacteria ($10^{8.55}$ bacteria instead of $10^{4.25}$).

TESTS FOR FORMATION OF PLAQUES ON FIBROBLAST CULTURES

Tests were conducted on 3T3 fibroblasts (ECA CC88031146) in accordance with the technique described by Kuhn et al., 1990 (5), except that the infections were made at varying concentrations of inoculum: 1 to 25 µl of 2-hour bacterial subcultures (A_{600nm} of 0.45), either undiluted or diluted to 1/10.

This test reveals the capability of Listeria monocytogenes to multiply intracellularly and to spread on monolayers of fibroblasts covered with a layer of agar containing gentamicin at a concentration that is lethal for extracellular bacteria but not for intracellular bacteria. After several days, areas of dead cells destroyed by the bacterial infection are visible to the naked eye in the form of "plaques."

The mutant bacteria LUT 12 were incapable of forming plaques on monolayers of 3T3 fibroblasts.

TEST OF SPREAD ON BONE MARROW MACROPHAGES

An observation under optical microscope of the spread of Listeria monocytogenes on monolayers of primary bone marrow macrophages was also made as follows.

Suspensions containing macrophages were prepared from bone marrow from a 7-week-old female C57BL/6 mouse and cultivated in an RPMI medium containing 10% fetal calf serum in the presence of supernatant from L. 4.10^5 macrophages derived from bone marrow obtained on day 6 were seeded [sic] on round glass slides (12mm in diameter) the night before they were used. The macrophages were infected with an MOI (multiplicity of infection) of 0.04 (one bacterium for 25 macrophages, resulting in approximately 1% infected cells), so as to be able to observe individual infection sites generated by the progeny of a single bacterium. The infection was produced as described for a J774 macrophage.

After 30 minutes and after 8 hours, those cellular monolayers were fixed and colored with Giemsa solution.

After 8 hours, the progeny of the wild-type bacteria had spread to many new host cells, and bacteria with protuberances at their end could be observed. In contrast, the progeny of the mutant LUT 12 remained enclosed inside a single infected cell. The mutant bacteria either formed microcolonies or spread in the cytoplasm of the host cells, but no protuberance containing bacteria could be detected.

This result indicates that the mutant bacterium multiplies inside the infected cells, but is incapable of infecting adjacent cells by spreading from cell to cell.

GROWTH TEST ON J774 MACROPHAGES

A test demonstrating that the mutant bacterium LUT 12 was capable of multiplying intracellularly was conducted by means of a growth test on J774 macrophages.

This test was conducted on J774 monolayers in 25-cm^3 plastic tissue culture flasks. The cells were infected with an MDI of 10 bacteria per cell. The intracellular bacterial count was calculated after 2, 6, and 10 hours of growth on the medium containing the gentamicin (5 µg/ml) by lysing the cellular monolayers, washing with cold distilled water, and spreading appropriate dilutions on plates containing a BHI medium.

After a 10-hour period, the growth curves of the wild-type bacteria and LUT 12 were identical.

OBSERVATIONS UNDER ELECTRON MICROSCOPE

The intracellular behavior of the mutant LUT 12 was observed under an electron microscope. J774 macrophages were infected with wild-type bacteria or the mutant strain for 30 minutes, followed by an incubation of 60 to 210 minutes in a medium containing the gentamicin. For the mutant and the wild type, free-living bacteria could be observed in the cytoplasm after 1 and 1/2 hours of infection. At that time, the wild type and the mutant were surrounded by a thin layer of crimped granular material, but only the wild type comprised filamentary material assembled on its surface, made up of actin filaments. At 4 hours after infection, the wild-type bacteria were surrounded by thick layers of F-actin filaments. In contrast, the mutant bacteria LUT 12 were almost naked. Even the fine crimped coating observed at the early stage of infection had disappeared.

To display the bacterial F-actin association in a specific manner, the authors made double fluorescent stains using FITC-phalloidin, a fungal toxin that binds to F-actin, and with an anti-L. monocytogenes serum, followed by a second antibody coupled to rhodamine to detect the bacteria. The J774 macrophages had been infected for 4 hours with the wild-type or mutant bacteria. While the wild-type bacteria were positively stained with the FITC-phalloidin, the mutant bacteria LUT 12, even though detectable with the anti-L. monocytogenes serum, remained invisible with the actin stain.

These results demonstrate that the bacteria LUT 12 escape from the phagosomes as effectively as the wild-type bacteria and multiply in the cytoplasm. Nevertheless, the mutant bacteria are never associated with the F-actin, are incapable of propagating inside the cell, and cannot infect neighboring cells by spreading directly. These observations suggest that the mutant LUT 12 is deficient in a component required for the process of forming actin filaments induced by Listeria monocytogenes.

B - DETERMINATION OF THE INSERTION POINT OF THE TRANSPOSON

This mutant was analyzed by Southern blot to determine the number of transposons inserted in its chromosome.

The chromosomal DNA was digested by Bam HI, Eco RO, Hind III, Kpn I, and Pst I.

Two different probes corresponding to Tn 917-lac were used (Shaw and Clewell, 1985 (11)): a probe covering 515 base pairs of the erythromycin-resistant gene, obtained by PCR with the oligonucleotides 5' -TTG GAA CAG GTA AAG GGC ATT TAA-3' (position 821 to 844) and 5' -AGT AAA CAG TTG ACG ATA TTC TCG-3' (position 1313 to 1336), and a probe covering the internal Hind III fragment of the transposon obtained by PCR with the oligonucleotides 5'-ACA ATT AAT GTC TCC CAT ATT -3' (position 3082 to 3102) and 5' -ACT GAT AAT TAA CCA AAA CAG-3' (position 4295 to 4315).

The transposon-chromosome junction was cloned from a chromosomal DNA bank obtained by restriction with Eco RI/Kpn I in pUC 18. A clone comprising an insertion segment, corresponding to the chromosome-transposon junction, was isolated and directly sequenced from the plasmid through the use of an oligonucleotide hybridizing with the right end of the transposon (5' -CTA AAC ACT TAA GAG AAT TG-3', position 5244 to 5263).

The transposon was inserted after adenine 497 in the nucleotide sequence of the Hind III - Eco RI fragment of the act A gene of the operon identified by Mengaud et al., 1991 (b) (8), of which the nucleotide sequence was described by Vazquez-Boland et al., 1992 (12).

The insertion point of the transposon Tn 917-lac is represented in the schematic drawing in Fig. (A).

The lecithinase-negative phenotype of the mutant LUT 12 is probably due to a polar effect of the mutation by insertion into act A, to the extent that the 3rd gene of the plcB operon encodes for lecithinase.

Additional studies have been carried out which have demonstrated that the loss of the polymerization activity of the actin was indeed due to a loss of expression of the act A gene.

Mutants were created by homologous recombination between the Listeria monocytogenes chromosome and fragments corresponding to parts of the plcB gene and open reading frames ORFX/Y and ORFZ (Fig. (A)), located downstream of the act A gene by insertion of plasmids at various sites.

Immunofluorescence studies using FITC-phalloidin and rhodamine staining of bacteria in infected J 774 macrophages showed that the plcB, ORFX/Y and ORFZ mutants were associated with F-actin filaments just like the wild-type bacteria. Those studies were supplemented by electron microscope studies that showed that those mutants were capable of stimulating the accumulation of actin in the same way as the wild type.

Consequently, those analyses show that mutations downstream of act A do not affect the accumulation of actin A and suggest that the incapacity of the mutant LUT 12 to polymerize the cellular actin is due to the absence of expression of the act A gene.

A transformation of the mutant LUT 12 carried out with act A shows, in addition, that the wild-type phenotype is restored, which excludes the possibility of a spontaneous mutation at another site on the chromosome.

These results thus demonstrate that the product of the act A gene is required for the accumulation of the Listeria monocytogenes actin and consequently for its pathogenic power.

The product of the act A gene was determined as described below:

III - Analysis of the product of the act A gene

The nucleotide sequence of the act A gene allows us to assume that that gene encodes for a 639-amino acid protein with a sequence signal and a membrane-spanning area (Vazquez Boland et al., 1992 (12)).

Additional studies were carried out on the one hand by a comparison of the surface proteins of wild-type Listeria monocytogenes and of the strain LUT 12.

The bacterial isolates were grown in 200 ml of brain-heart infusion broth (BHI, DIFCO Laboratories, Detroit, Michigan), to which was added erythromycin at 5 µg/ml for LUT 12, shaken at 160 tpm on a Gyrotory G10 shaker (New Brunswick Scientific) at 37°C for 18 hours.

The bacteria were gathered by centrifugation (5,000 g for 20 minutes) and washed three times in phosphate-buffered saline (PBS).

The pellet obtained was suspended in 4 ml of PBS and SDS was added to a final concentration of 1%. At this concentration of SDS, the L. monocytogenes cells do not lyse. The absence of bacterial lysis was verified under a microscope. After 5 minutes of shaking at room temperature, the bacteria were centrifuged (50,000 g for 10 minutes) and the supernatant concentrated by ultrafiltration in microconcentrators (Centricom 30, Amicon) and kept at -20°C.

The protein concentration was determined using the bicinchonic acid method (Pierce). The protein concentration was adjusted to 300 µg/ml for the electrophoresis. 10 µl of extract was mixed with 10 µl of buffer (2% SDS, 10% glycerol, 5% mercaptoethanol, 0.002% bromophenol blue, and 0.02 M Tris HCl), boiled for 3 minutes at 100°C. The electrophoresis was carried out at 60 mA for 120 minutes through polyacrylamide freeze-thaws (Laemmli, 1970 (6)). The bands were displayed by silver staining (Heukeshoven and Dernick, 1985 (4)).

To mark the cellular surface, 400 ml of an 18-hour Listeria monocytogenes culture were centrifuged; the bacteria were washed 3 times with PBS at pH 7.4 and suspended in 8 ml of PBS, pH 8.0, at 4°C.

The bacteria were then treated with N- hydroxysulfosuccinimide-biotin (sulfo-NHS-biotin; Pierce) at a final concentration of 0.5 mg/ml for 2 minutes under moderate shaking.

The cells were washed three times with PBS at pH 7.4 and extracted by SDS extraction.

The extracts corresponding to 7 µg of proteins per channel were deposited on SDS gels and transferred as described by De Rycke et al., 1989 (2) on cellulose nitrate (BA 85, Schleicher and Schüll). The cellulose nitrate filters were saturated for one night in PBS with 0.5% gelatin and incubated for 1.5 hours with streptavidin conjugated to peroxidase (Jackson) in PBS containing 0.5% gelatin and 0.1 M of Tween 20. After several washes in the same buffer, the reactive bands were revealed with 0.5 mg/ml of 4-chloro-1-naphthol (Biorad) and 0.03% v/v of H_2O_2 in water.

The analyses of the electrophoresis gels show a 90 kDa band for the wild type which is absent in the strain LUT 12. This band is also found in the plcB mutants and the mutants LUT 12 transformed by act A noted above.

The analyses of surface marking by the N- hydroxysulfosuccinimide-biotin directly show a 90 kDA biotinylated protein in the wild-type bacteria that is absent in the mutant strain LUT 12.

To unambiguously identify the 90 kDA protein, the 90 kDA band was isolated and the sequence of 6 amino acids of the NH_2 end was determined and compared with the amino acid sequence deduced from the nucleotide sequence of the act A gene.

The extracts on SDS corresponding to 100 µg of proteins per channel were brought to a boil in an SDS sample buffer containing 7% (p/v) urea before carrying out electrophoresis on gels with 7.5% SDS.

The separated proteins were transferred to a Problott membrane (Applied Biosystems) in 50 mM Tris - 50 mM borate for 17 hours at 4 to 5 volts/cm. The proteins were stained for 5 seconds with 0.1% amido black in a 1% acetic acid and 40% methanol solution and carefully rinsed with water. A 90 kDa band was cut from several channels. The proteins of the membrane were sequenced by degradation in accordance with Edman in a 740 A Applied Biosystems sequencer with, on-line, a 120 A HPLC PTH analyzer programmed for the Problott membrane by the manufacturer. The amino acid sequences were analyzed on a Data General MV 10000 computer at the Scientific Data Processing Unit of the Pasteur Institute.

The Ala-Thr-Asp-Ser-Glu-Asp sequence of the isolated protein corresponds exactly to the amino acids of the cleavage site of the predicted signal sequence according to the peptide sequence predicted on the basis of the act A gene (Fig. B).

Consequently, the mature product of the act A gene is a 610-amino acid protein with a calculated molecular weight of 67 kDA. It has an apparent molecular weight of 90 kDA and is expressed on the surface of the bacterium.

This protein is required for the accumulation of F-actin and its absence leads to a very significant attenuation of the virulence of Listeria monocytogenes. Consequently, any mutation that affects the act A gene or its promoter and appreciably modifies or prevents the expression of its product, makes it possible to obtain a non-pathogenic attenuated strain in accordance with the invention.

The results obtained in vivo with the strain LUT 12 on the protection of mice against a Listeria monocytogenes infection are reported below.

IV - In vivo effects of the strain LUT 12: study in mice

A/ Multiplication of the actA mutant in the liver and spleen of infected mice.

The behavior of LUT 12 was studied, after intravenous injection, in the spleen and liver of mice, which are the principal target organs where wild-type Listeria monocytogenes express their pathogenicity. The clinical tests used were the following:

the livers and spleens of infected mice were collected at various times after the infection and homogenized to allow the release of bacteria and the living bacteria were counted in vitro.

The LD_{50} of the actA mutant LUT 12, after intravenous injection in pure line C3H mice was higher by a factor of 3 $\log_{10}$ than that of wild-type Listeria monocytogenes (2.5×10^7 compared to 2.5×10^4).

The growth kinetics of the actA mutant and of the virulent wild-type strain in the liver and spleen were compared. After intravenous injection of a maximum sublethal dose of the actA mutant (1.5×10^7 organisms) or of two different doses of virulent L. monocytogenes (7×10^3 or 6×10^4), the bacterial count in the liver and spleen of the infected mice was determined at variable times in the course of the infection.

An increase in the number of actA mutants was observed in the spleen during the first 24 hours, but this increase was limited (1 $\log_{10}$) in comparison with the increase by a factor of 4 $\log_{10}$ observed with the wild-type strain. From day 1, the number of actA mutant bacteria rapidly decreased and on day 5, almost no bacteria could be collected from the spleen. In contrast, the wild-type strain of Listeria monocytogenes could still be detected in that organ on days 9 to 10 of the infection. In the liver, the number of actA mutant bacteria continued at a stable level until day 4 and after that, decreased rapidly; in contrast, the number of wild-type Listeria monocytogenes increased by a factor of 2 $\log_{10}$ before reaching a plateau on days 6 to 7.

The persistence of the actA mutant at a stable level in the liver for 4 days may reflect either a balance between bacterial multiplication and bacterial death, or a survival of the bacteria without multiplication. To discriminate between those two possibilities, the bacterial growth curves in the liver and spleen of mice treated with ampicillin were compared to those of control mice. Ampicillin inhibits the synthesis of peptidoglycan and is a bactericide on bacteria in an active multiplication stage. The infected mice were treated on two occasions with 15 mg of ampicillin administered intraperitoneally from day 1, 2, or 3 of the infection; the liver and spleen were removed one day later and the number of remaining bacteria was determined and compared with that obtained from mice not treated with the antibiotic.

After such treatment, the number of actA bacteria decreased abruptly in the spleen on day 2 and in the liver on days 2 and 3, but no difference between the control growth curve [sic] was found on days 3 and 4 in the spleen or on day 4 in the liver.

These results suggest that the persistence of the actA mutant in the liver is due to a balance between the bacterial multiplication and death. Given that the actA mutants are deficient with respect to the spread from cell to cell in vitro, the persistence of actA in the

liver is probably due to an infection in the neighboring cells after lysing of the first infected host cells; consequently, Listeria monocytogenes may be exposed to bacterial effectors present in an extracellular environment and its capacity to spread locally may be diminished.

In addition, if extracellular Listeria monocytogenes are phagocytized by macrophages activated by interferon γ, they may be incapable of reaching the cytosol and continuing their intracellular cycle.

Finally, the actA mutants were eliminated from the spleen and liver earlier than the wild-type strain, suggesting that the protective effectors of the host are quickly induced in mice infected with the actA mutant.

B/ Effects of a single infection with the actA mutant on the induction of persistent immunity.

The existence of a non-specific resistance due to the activation of macrophages is a well known phenomenon, occurring rapidly and temporarily in mice "recovering" from a sublethal infection.

For the purpose of avoiding the simultaneous detection of non-specific and specific immunity effects, the inventors determined at what point in time the non-specific resistance ceased to be expressed.

They intravenously injected an unrelated intracellular pathogen, Yersinia enterocolitica Ye8081 0:8 (16, 17), into either naïve mice or mice infected with the actA mutant 4, 6.5, and 8.5 weeks before the injection. They compared the bacterial count in the spleen and liver in these two groups of mice. No difference was observed between the two groups at each point of the test. They then carried out the following experiments 6 weeks or more after the infection with the actA mutant.

First, the LD_{50} of wild-type L. monocytogenes was determined in mice that had been infected 6 weeks earlier with the actA mutant and in control mice: a difference on the order of 100 was observed between the two groups (2.2×10^6 and 2.5×10^4 respectively).

Second, the growth curves of wild-type L. monocytogenes were compared in the liver and spleen of naïve mice and mice immunized 6 weeks earlier, during the first 3 days of the infection. A significant slowing of bacterial growth was observed from day 1 in the spleen and from days 2 to 3 in the liver.

Third, this specific inhibition of the growth of wild-type L. monocytogenes was still effective 8.5 weeks after infection with the actA mutant (decrease of 4.01 $\log_{10}$ in the bacterial count in the spleen 48 hours after a bacterial inoculum of 5×10^4).

These results show that a single infection with the attenuated actA mutant is sufficient to induce immunity against wild-type L. monocytogenes.

C/ Generation of $CD8^+$ T lymphocytes that protect against Listeria.

A transfer of protection was carried out in naïve syngeneic "recipients" by using spleen cells collected 7 days after an intravenous injection of 1.5×10^7 actA mutant bacteria. The "recipients" were exposed to an intravenous infection with a lethal dose of wild-type L. monocytogenes for one hour and the bacterial count was determined in the liver and spleen of the "recipients" two days after the infection.

First, the injection of splenocytes from mice infected with the actA mutant caused a significant decrease in the number of wild-type L. monocytogenes collected from the spleen of the "recipients" that received the cells and this effect was dose-dependent. The decrease in the bacterial count was also observed in the liver of the "recipients," but to a lesser degree (decrease of 1.00 ± 0.45 $\log^{10}$, $n = 4$, for transfers of 5×10^7 to 2.5×10^8 cells, $P < 0.02$).

For the purpose of characterizing the phenotype of the protective spleen cells, in the immunized spleen cell population, we removed either Thy-1^+, $CD4^+$, or $CD8^+$ lymphocytes, before the passive transfer (Table 1 below). The transfer of the splenocytes that were not removed resulted in a reduction of 3 to 4 $\log_{10}$ in the bacterial count in the spleen. This protection was transferred by the T lymphocytes because a depletion in Thy-1^+ lymphocytes eliminated the decrease in the bacterial load in the spleen. The high level of protection provided by immune splenocytes after 7 days was only slightly affected by the depletion of the CD4 sub-population. Most of the protective effect provided by the immune splenocytes after 7 days was sensitive to a depletion of the CD8 sub-population, but could not be attributed solely to the sub-population of $CD8^+$ lymphocytes.

To the extent that the Thy-1 depletion suppressed the protection and that the CD4 depletion had only a marginal effect, we may consider that a part of the protective role not dependent on CD8 is due to double-negative T lymphocytes, as has already been observed by DUNN and NORTH (18). Previously published experiments of depletion/protection on lymphoid cells from mice recovering from an infection with wild-type L. monocytogenes showed that wild-type L. monocytogenes was capable of inducing a protection that was almost exclusively due to $CD8^+$ lymphocytes and that those $CD8^+$ lymphocytes provided protection without the participation of $CD4^+$ lymphocytes.

The actA mutant of L. monocytogenes is thus capable of inducing the production of specific $CD8^+$ lymphocytes that protect against Listeria.

The actA mutant has a functional gene of the listeriolysin-O which allows it to escape from the phagosome and enter the cytosol; it is likely that the actA mutant is capable of stimulating the production of $CD8^+$ T lymphocytes that protect against Listeria, recognizing natural peptides of L. monocytogenes. In addition, the capacity of actA mutant to multiply temporarily in the organs of infected mice and to secrete a sufficient quantity of bacterial proteins is probably critical to making possible an effective production of protective $CD8^+$ lymphocytes.

The Tn917-lac transposon is inserted in actA, the second gene of the lecithinase operon, and has a polar effect on the expression of the plcB gene that encodes for a lecithinase. The results obtained in this invention indicate that lecithinase does not play an essential role in inducing protective immunity against L. monocytogenes.

In conclusion, the results of this invention show that attenuated actA mutants are capable of inducing $CD8^+$ T lymphocytes that protect against Listeria and that a protective immune state against wild-type L. monocytogenes can be established by a single infection. As the actA mutants penetrate into the cytosol of the infected cells and multiply in that compartment, we can consider their use as a living vector to deliver heterologous proteins into the cytosol and to promote the production of $CD8^+$ T lymphocytes; such living vectors capable of multiplying temporarily are assumed to deliver a sufficient load of heterologous proteins into the cytosol for a short time period. In addition to their potential use as a model for the development of vaccines that use living vectors, these Listeria of attenuated virulence may be useful for screening and characterizing specific bacterial or parasitic peptides of alleles of the locus that encodes for class 1 MHC molecules. Indeed, certain bacteria and certain parasites reside in the vacuolar compartments; in addition, the growth kinetics may be weak. Thus, for the Leishmania species or the Mycobacterium species, this tool could be very useful for defining the specificity of the $CD8^+$ lymphocytes that are produced in response to the infection.

Induction of the "propagation of protective $CD8^+$ T lymphocytes" by the actA mutant of L. monocytogenes in the spleen of C3H mice

Experiment	Experiment 1 (5×10^7 cells)	Experiment 2 (1×10^8 cells)	Experiment 3 (2×10^8 cells)
Cell transfer	- + + +	- + + + +	- + + +
In vitro transfer	- C' anti-CD4 + C' anti-CD8 + C'	- C' anti-THyl + C' anti-CD4 + C' anti-CD8 + C'	- C' anti-CD4 + C' anti-CD8 + C'
Listeria $\log_{10}$ per cell	6.70 ± 0.14 4.00 ± 0.19 4.60 ± 0.45 6.25 ± 0.39	6.51 ± 0.16 2.53 ± 0.32 6.16 ± 0.20 3.59 ± 0.34 4.89 ± 0.64	6.75 ± 0.21 2.51 ± 0.45 3.20 ± 0.32 5.36 ± 0.25
VALUE of P Treated cells versus no cells	- < 0.001 < 0.001 < 0.05	- < 0.001 NS < 0.001 < 0.01	- < 0.001 < 0.001 < 0.001
Cells removed versus cells treated with C'	- - NS < 0.001	- - < 0.001 < 0.05 < 0.01	- - NS < 0.001

The spleen cells were isolated from C3H mice on day 7 after intravenous injection of 1.5 x 10^7 actA mutants. The Thy-1^+, $CD4^+$, or $CD8^+$ cells were removed in vitro before the adoptive transfer. The monoclonal antibodies used here were anti-Thy-1, 2 Jlj (ATCC TIB 184) anti-CD4 RL1724 (*Ceredig, R. Lowenthal, J.W. Nabholz M. and MacDonald, H.R., 1985, Nature 314 98*) and anti-CD8 31M (*Sarmiento M. Glasebrook A.L. and Fitch F.W., 1980, J. Immunol. 125 - 2665*). The depletion was < 90% after each cytotoxic treatment by monoclonal antibodies (results not shown). The non-depleted [complement (C')] or depleted cellular populations were transferred to syngeneic naïve "recipients" one hour after injection of 4 to 8 x 10^4 wild-type L. monocytogenes. The control groups comprised recipients of non-depleted cells (i.e., incubated only with C') and recipients receiving only one injection of wild-type L. monocytogenes (i.e., no cells). 48 hours after the injection, the bacteria were counted (average + ET) in the spleens of the "recipients" (3 to 5 mice per group). The statistical significance (Student test) is reported for the effectiveness of the transfer (cells treated versus no cells) and for the effect of the depletion treatment on the effectiveness of the transfer (depleted cells versus cells treated with C').

CLAIMS

1. Attenuated mutant of Listeria monocytogenes comprising, in the act A gene or in the act A gene promoter, a mutation capable of blocking or appreciably modifying the expression of the protein encoded by the act A gene.

2. Attenuated mutant of Listeria monocytogenes as claimed in claim 1, characterized by the fact that the mutation consists of an insertion, a deletion, or a mutation by directed mutagenesis.

3. Attenuated mutant of Listeria monocytogenes as claimed in claim 1 or claim 2, characterized by the fact that the mutation consists of the insertion of a stable transposon.

4. Attenuated mutant of Listeria monocytogenes as claimed in claim 3, characterized by the fact that the stable transposon is the Tn917-lac transposon.

5. Attenuated mutant of Listeria monocytogenes as claimed in any of the preceding claims, characterized by the fact that the mutation is carried out in the DNA fragment that encodes for the peptide sequence with repeated patterns included between amino acids 235 to 315, 350 to 360, 367 to 385, and 389 to 393 of sequence SEQ ID No. 1.

6. Attenuated mutant as claimed in one of claims 1 to 4, characterized by the fact that the mutation consists of an insertion between amino acids 61 and 62 of peptide sequence SEQ ID No. 1.

7. Attenuated mutant of Listeria monocytogenes as claimed in claim 6, named LUT 12, deposited in the CNCM on January 30, 1992 under the number I-1167.

8. Human or veterinary vaccine, characterized by the fact that it comprises as an active ingredient an attenuated mutant strain of Listeria monocytogenes as claimed in one of the preceding claims.

9. Recombinant strain of Listeria monocytogenes characterized by the fact that it comprises a heterologous DNA, either inserted in the genome of an attenuated mutant as claimed in one of the preceding claims, or carried by a plasmid that replicates in the attenuated mutant.

10. Recombinant strain as claimed in claim 9, characterized by the fact that the heterologous DNA consists of a heterologous gene that encodes for a protective antigen that is the target of sub-class CD8 T lymphocytes.

11. Recombinant strain as claimed in claim 10, characterized by the fact that the antigen is a bacterial antigen, in particular of mycobacteria.

12. Recombinant strain as claimed in claim 10, characterized by the fact that the antigen is a parasitic antigen, in particular of Leishmania, Trypanosoma, or Toxoplasma, Theileria.

13. Recombinant strain as claimed in claim 10, characterized by the fact that the antigen is a viral antigen, in particular of HIV, of the lymphocytic choriomeningitis virus, or of the influenza virus.

14. Recombinant strain as claimed in claim 13, characterized by the fact that the antigen is the gag antigen and/or the nef antigen of HIV and/or all or part of the HIV-1 coating gp 120 or HIV-2 coating gp 140.

15. Recombinant strain as claimed in one of claims 9 to 14, characterized by the fact that it comprises a promoter of Listeria upstream of the heterologous DNA.

16. Recombinant strain as claimed in claim 15, characterized by the fact that the promoter is the hly promoter.

17. Recombinant strain as claimed in claim 16, characterized by the fact that the heterologous DNA is fused with the beginning of the hly gene in order to use the signal sequence of the listeriolysin O to secrete the product of the heterologous DNA in the cytoplasm of the host cell.

18. Recombinant human or veterinary vaccine, characterized by the fact that it comprises as an active ingredient a recombinant strain as claimed in one of claims 9 to 17.

19. Diagnostic composition comprising a recombinant strain of Listeria monocytogenes as claimed in one of claims 10 to 17, for checking the protection status of a human or animal host against an infection caused by a micro-organism that comprises an antigen roughly identical to that encoded by the heterologous gene inserted in the recombinant mutant strain or carried by a plasmid that replicates in the recombinant mutant strain.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR93/00105

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.^{5} : C12N 15/74; C12N 1/21; C12N 15/01; A61K 39/02 //C12N 15/31

According to International Patent Classification (IPC) or to both national classification and IPC (C12N 1/21, C12R1:01)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl.^{5} : C12N; C07K; A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INFECTION AND IMMUNITY. Vol. 58, No.11, November 1990, WASHINGTON US pages 3770-3778 A.N. SUN ET AL 'Isolation of listeria monocytogenes Small-plaque mutants defective for intracellular growth and cell-to-cell spread' *see the whole document in particular page 3775 left-hand column*	1-4
A	INFECTION AND IMMUNITY. Vol. 60, No.1, January 1992, WASHINGTON US pages 219-230 JOSE-ANTONIO VAZQUEZ-BOLAND ET AL 'Nucleotide sequence of the lecithinase operon of Listeria monocytogenes and possible role of lecithinase in cell-to-cell spread' cited in the application see page 226, right-hand column see page 228, right-hand column see abstract	1-4
A	INFECTION AND IMMUNITY ./.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 June 1993 (23.06.93)	13 July 1993 (13.07.93)
Name and mailing address of the ISA/ European Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR93/00105

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Vol. 58, No.11, November 1990, WASHINGTON US pages 3477-3486 M. KHUN ET AL 'A non-virulent mutant of Listeria monocytogenes does not move intracellularly but still induces polymerization of actin' cited in the application see the whole document	
P,X	CELL. Vol. 68, 7 February 1992, CAMBRIDGE, NA US pages 521-531 C. KOCKS ET AL 'L. monocytogenes - induced Actin assembly requires the ActA gene product, a surface protein' see the whole document	1-19
P,X	EMBO JOURNAL. Vol. 11, No.5, May 1992, EYNSHAM, OXFORD GB pages 1981-1990 E. DOMANN ET AL 'A novel bacterial virulence gene in Listeria monocytogenes required for host cell microfilament interaction with homology to the proline-rich region of vinculin' see the whole document	1-2
A	MOLECULAR MICROBIOLOGY Vol. 5, No.9, 1991, pages 2273-2283 MENGAUD J. ET AL 'Pleiotropic control of Listeria monocytogenes virulence factors by a gene that is autoregulated' cited in the application see the whole document	1-4
A	JOURNAL OF BACTERIOLOGY Vol. 174, No.2, January 1992, pages 568-574 TRINAD CHAKRABORTY ET AL 'Coordinate regulation of virulence genes in Listeria monocytogenes requires the product of prfA gene' *see the whole article, especially pages 571 right-hand column lines 28-31, page 572 lines 41-49 and page 573 lines 3-10*	1-2

RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale No PCT/FR 93/00105

I. CLASSEMENT DE L'INVENTION (si plusieurs symboles de classification sont applicables, les indiquer tous) [7]

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

CIB 5 C12N15/74; C12N1/21; C12N15/01; A61K39/02
//C12N15/31(C12N1/21,C12R1:01)

II. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée[8]

Système de classification	Symboles de classification
CIB 5	C12N ; C07K ; A61K

Documentation consultée autre que la documentation minimale dans la mesure où de tels documents font partie des domaines sur lesquels la recherche a porté[9]

III. DOCUMENTS CONSIDERES COMME PERTINENTS[10]

Catégorie *	Identification des documents cités, avec indication, si nécessaire,[12] des passages pertinents[13]	No. des revendications visées [14]
X	INFECTION AND IMMUNITY. vol. 58, no. 11, Novembre 1990, WASHINGTON US pages 3770 - 3778 A. N. SUN ET AL 'Isolation of listeria monocytogenes Small-plaque mutants defective for intracellular growth and cell-to-cell spread' * le document en entier plus particuliérement page 3775 colonne de gauche * --- -/--	1-4

* Catégories spéciales de documents citées:[11]

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié postérieurement à la date de dépôt international ou à la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier.

"&" document qui fait partie de la même famille de brevets

IV. CERTIFICATION

Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
23 JUIN 1993	13.07.93
Administration chargée de la recherche internationale	**Signature du fonctionnaire autorisé**
OFFICE EUROPEEN DES BREVETS	LE CORNEC N.D.R.

III. DOCUMENTS CONSIDERES COMME PERTINENTS [14] (SUITE DES RENSEIGNEMENTS INDIQUES SUR LA DEUXIEME FEUILLE)

Catégorie *	Identification des documents cités, [16] avec indication, si nécessaire des passages pertinents [17]	No. des revendications visées [18]
A	INFECTION AND IMMUNITY. vol. 60, no. 1, Janvier 1992, WASHINGTON US pages 219 - 230 JOSÉ-ANTONIO VAZQUEZ-BOLAND ET AL 'Nucleotide sequence of the lecithinase operon of Listeria monocytogenes and possible role of lecithinase in cell-to-cell spread' cité dans la demande voir page 226, colonne de droite voir page 228, colonne de droite voir abrégé	1-4
A	INFECTION AND IMMUNITY. vol. 58, no. 11, Novembre 1990, WASHINGTON US pages 3477 - 3486 M. KUHN ET AL 'A non-virulent mutant of Listeria monocytogenes does not move intracellularly but still induces polymerization of actin' cité dans la demande voir le document en entier	1-4
P,X	CELL. vol. 68, 7 Février 1992, CAMBRIDGE, NA US pages 521 - 531 C. KOCKS ET AL 'L. monocytogenes-induced Actin assembly requires the ActA gene product , asurface protein' voir le document en entier	1-19
P,X	EMBO JOURNAL. vol. 11, no. 5, Mai 1992, EYNSHAM, OXFORD GB pages 1981 - 1990 E. DOMANN ET AL 'A novel bacterial virulence gene in Listeria monocytogenes required for host cell microfilament interaction with homology to the proline-rich region of vinculin' voir le document en entier	1-2
A	MOLECULAR MICROBIOLOGY vol. 5, no. 9, 1991, pages 2273 - 2283 MENGAUD J. ET AL 'Pleiotropic control of Listeria monocytogenes virulence factors by a gene that is autoregulated' cité dans la demande voir le document en entier	1-4

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III. DOCUMENTS CONSIDERES COMME PERTINENTS [14] (SUITE DES RENSEIGNEMENTS INDIQUES SUR LA DEUXIEME FEUILLE)

Catégorie *	Identification des documents cités, [16] avec indication, si nécessaire des passages pertinents [17]	No. des revendications visées [18]
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